PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/00, 5/02, C12Q 1/68, 1/66, 1/02, A61K 31/70 — A1

(11) International Publication Number: WO 94/17177

(43) International Publication Date: 4 August 1994 (04.08.94)

(21) International Application Number: PCT/US94/00973

(22) International Filing Date: 27 January 1994 (27.01.94)

(30) Priority Data:
08/009,827 27 January 1993 (27.01.93) US

(71) Applicant: BATTLE LUMINETRICS ENTERPRISES, INC. [US/US]; 5601 North Dixie Highway, Suite 215, Ft. Lauderdale, FL 33334 (US).

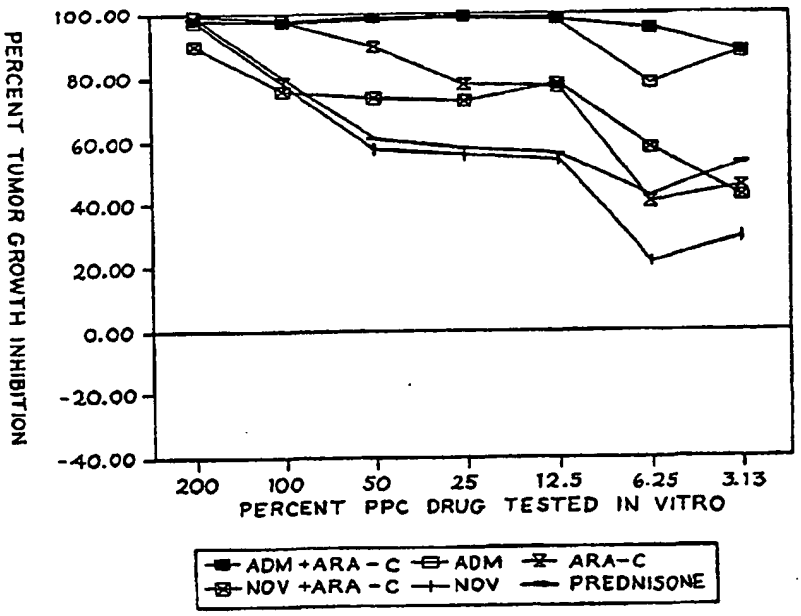
(72) Inventor: ANDREOTTI, Peter, E.; 3940 N.W. 75 Ter., Lauderhill, FL 33319 (US).

(74) Agent: MARSCHANG, Diane, L.; Willian, Brinks, Hofer, Gilson & Lione, NBC Tower, Suite 3600, 455 North Cityfront Plaza Drive, Chicago, IL 60611 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHODS AND KITS FOR DETERMINING EFFECTS OF ANTI-CANCER AGENTS ON CANCER CELLS



(57) Abstract

Methods for performing in vitro assays to detect and quantitate the effects of anti-cancer agents on cancer cells are provided. In the methods of the invention, mammalian cancer cells are cultured in the presence of a single anti-cancer drug agent, or a combination of anti-cancer drug agent, in culture medium free of heterologous serum. After culturing the cells with the anti-cancer drug agent, Atp from the cells is extracted and stabilized, and measured using luminescence techniques, as illustrated in the figure. Kits for performing in vitro assays to detect and quantitate the effects of anti-cancer agents on cancer cells are also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SI	Slovenia
SK	Slovakia
SN	Senegal
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

METHODS AND KITS FOR DETERMINING EFFECTS OF ANTI-CANCER AGENTS ON CANCER CELLS

FIELD OF THE INVENTION

The invention relates to methods and kits for determining the effects of anti-cancer agents on cancer cells using ATP luminescence techniques.

RELATED PATENT APPLICATIONS

This application is a continuation-in-part application of application U.S. Serial No. 07/701,870, filed May 17, 1991, the contents of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

It is established that over 1 million people will be diagnosed with cancer in 1992. [Cancer Facts & Figures, American Cancer Society (1992)]. While some therapeutic protocols have been shown useful in the treatment of cancer, the efficacy of those treatment protocols varies among patients. This is believed to be due not only to the diversity among different types of cancers and tumors, but to drug resistance and the variability among patients in their response to different therapies.

To assist in formulating new types of cancer therapy, and to better predict which type of therapy may be effective for individual patients, a variety of in vitro assays have been developed. Previous assays for testing the effects of certain cancer therapy on cancer cells include what are known in the art as "clonogenic" assays. [See, e.g., U.S. Patent No. 4,411,990]. Clonogenic assays are typically

performed by cloning cancer cells in agar after exposure to particular anti-cancer drugs. These assays, however, have several disadvantages. For instance, their usefulness is limited since some cancer cells, particularly some leukemic cells and solid tumor cells, are unable to grow and form colonies in agar cultures. Further, the assays can take as long as two to three weeks to conduct. The results of the clonogenic assays are then evaluated primarily on subjective criteria, such as colony size and numbers of colonies. These subjective criteria can lead to increased variability of results and statistically inaccurate data. The overall accuracy of clonogenic assays has also been criticized because it is believed that the proliferating stem cells which are measured in such assays may not be the only important target cells for determining sensitivity or resistance to cancer therapy.

Other types of in vitro assays for testing the effects of certain cancer therapy on cancer cells, referred to as "nonclonogenic" assays, include enzyme colorimetric assays [Santini et al., Hematological Oncology, 7:287-293 (1989)], thymidine incorporation assays [Kern et al., Cancer Research, 45:5436-5441 (1985)], and differential staining and fluorescence assays [U.S. Patent No. 4,816,395; U.S. Patent No. 4,996,145; Rotman et al., In Vitro Cellular & Developmental Biology, 24:1137-1146 (1988)]. More particularly, U.S. Patent No. 4,559,299 discloses a method for predicting responsiveness of abnormal cells to cytotoxic agents by measuring the retention of fluorescein or other label by cell membranes. The reference teaches that the responsiveness of cancer cells to cytotoxic agents can be evaluated by monitoring changes in the rate of fluorescein released by the cultured cells. An apparatus for conducting the

fluorescence assay is disclosed in U.S. Patent No. 4,734,372.

U.S. Patent No. 4,937,187 also discloses a fluorescence assay utilizing cultured tumor cell aggregates which retain some of the three-dimensional structure or organization of the original tumor. In the disclosed assay, tumor fragments are suspended in an aqueous medium so that non-aggregated cells and cellular debris form a supernatant and the aggregated tumor cells deposit into a sediment layer. The resulting supernatant is decanted and discarded, and the aggregates are cultured in media containing fetal bovine serum. To measure cell fluorescence, the culture medium is removed and a fluorescein monoacetate solution (diluted in serum-free RPMI media) is added. After measuring the fluorescence, media containing fetal bovine serum is again added to the culture. According to Rotman et al., In Vitro Cellular & Developmental Biology, 24:1137-1146 (1988), exposure of the fluorogenic substrate is performed in serum-free medium since protein concentrations above 10 mg/ml interfere with fluorochromasia.

While the nonclonogenic assays offer some advantages over clonogenic assays, they have encountered some technical problems. For example, nonclonogenic assays typically involve growth of the cancerous cells as well as growth of non-cancerous cells that may be present in the sample. It has been reported that one of the major obstacles in designing an in vitro assay for determining sensitivity of cancer cells to drugs is the problem of measuring specific cytotoxic effects on the cancer cells in the presence of non-malignant, or normal, cells present in the cultures. [Rotman et al., In Vitro Cellular & Developmental Biology, 24:1137-1146 (1988)].

Also, nonclonogenic assays utilizing radioisotopes [See, e.g., U.S. Patent No. 4,806,464;

U.S. Patent No. 5,030,417] tend to be expensive and hazardous, and typically require active cell division for isotope incorporation. This is problematic because some cancers, like breast carcinomas and leukemias, often contain a high percentage of non-proliferating cells [Zeigler et al., Cancer, 68:628-633 (1991)] and many cancer cells in the cell culture may be in the G_0 inactive stage of the cell cycle.

The use of certain luminescence techniques overcomes many of these problems. Generally, the term "luminescence" covers a wide range of processes that produce light. One type of luminescence technique is known as fluorescence. Generally, fluorescence is a luminescence technique that requires an external light source to excite fluorescein labels or other fluorescent compounds.

Chemiluminescence and bioluminescence are two other specific types of luminescence. Chemiluminescence is light emission that arises during the course of a chemical reaction, while bio-luminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. Light is produced when molecules, formed in an electronically excited state, decay to what is known as the ground state.

One particular type of bioluminescence reaction involves the firefly bioluminescence reaction. This light-producing reaction, which requires the enzyme firefly luciferase, D-luciferin, Mg^{2+}, ATP and molecular oxygen, is shown in the following equation:

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow[Mg2+]{\text{Luciferase}} \text{AMP} + 2\ \text{Pi} + CO_2 + \text{light}$$

The overall reaction is an oxidation reaction catalyzed by the enzyme, luciferase, which results in the emission of light.

The use of bioluminescence and chemiluminescence reactions in assays offers several advantages, including relatively high sensitivity, rapid results, low costs, and objective measurements using simple and inexpensive equipment. In addition, measuring the luminescence of cellular ATP in such assays allows for the evaluation of a number of cells from both dividing and non-dividing cell populations.

Among the prior art assays utilizing bioluminescence and chemiluminescence techniques are assays for detecting HLA antigens and anti-HLA antibodies [U.S. Patent No. 4,314,026]; quantitating microorganisms in a sample [U.S. Patent No. 3,933,592; U.S. Patent No. 3,745,090]; and quantitating susceptibility of bacteria to antimicrobial agents [U.S. Patent No. 4,014,745]. [See also, Kricka et al., Analytical Applications of Bioluminescence and Chemiluminescence, Academic Press, pp. 286-287 (1984)].

U.S. Patent No. 4,303,752 also discloses a method for measuring ATP luminescence. More particularly, the reference discloses a method for extracting ATP from somatic and microbial cells using a surface active agent. The reference teaches that non-ionic surface active agents may be used to release ATP from cells without the complete destruction of the cell membrane, but that the ATP extracted by those methods should be measured within about 5 minutes (Col. 6, lines 58-68; Col. 11, lines 28-32) since the ATP is not stabilized in the solution.

Kangas et al., Medical Biology, 62:338-343 (1984), describe methods for evaluating drug effects on cancer cells by measuring ATP luminescence. In particular, the authors report the effects of cytotoxic compounds on rat Walker 256 carcinoma cell line, MCF-7 cell line, and an ovarian serous cystadenocarcinoma cultured in medium containing 10% fetal calf serum. The reference teaches that ATP was released from the

cultured cells using trichloroacetic acid or NRS reagent, and then measured by luminometry.

Garewal et al., J. Natl. Cancer Inst., 77:1039-1045 (1986), report an ATP luminescence assay for measuring cytostatic and cytocidal drug effects on the human cancer cell lines, WiDR and T-47. According to the reference, both cell lines were grown and maintained in McCoy's 5-A medium supplemented with 10% fetal calf serum. Sevin et al., Gynecol. Oncol., 31: 191-204 (1988), also describe an ATP luminescent assay for measuring the effects of chemotherapy drugs on cancer cells cultured in a fetal calf serum-containing medium.

As discussed above, in vitro assays involving the culturing of cancer cells have been used for determining the efficacy of cancer therapy on cancer cells, and for determining appropriate therapies for individual patients. To assure the accuracy of such culture assays, however, effective tissue culturing techniques are important. Cells are typically cultured in vitro in tissue culture media that contains, among other things, serum such as fetal calf serum, fetal bovine serum, or horse serum. The serum supplement is usually considered necessary to the survival of the cell culture since the serum includes exogenous growth factors used to promote cell growth.

Although serum-free tissue culture medias have previously been described, their use in in vitro assays has been limited. Simms et al. have reported that a cell line of human small cell (Oat Cell) carcinoma of the lung (NCI-H69) is able to replicate in serum free medium. [Simms et al., Cancer Research, 40: 4356-4363 (1980)].

U.S. Patent No. 5,063,157 discloses a serum-free culture medium for growing mammalian cells, particularly, non-adherent cells, in vitro. The reference discloses a medium that comprises a base

medium, preferably DMEM/F12, along with other supplements such as insulin and transferrin. The reference teaches that the serum-free medium is cost effective and reduces problems associated with cell aggregation.

Investigators have reported that cytokines produced in vivo by normal cells, and by cancer cells themselves, may have regulatory, or autocrine control on growth of cancer cells. [See, Miles et al., Science, 255:1432-1434 (1992)]. More particularly, Nakazaki, Cancer, 70:709-713 (1992), describes the changes in TNF-α, IL-1α, IL-1β, IL-6, and GM-CSF levels in cancer patients, before and after surgical removal of the cancer. Nakasaki reports that serum levels of all these cytokines are high in patients with cancer, and that patients with postoperative cancer recurrence exhibited higher levels of cytokines than did those patients without recurrence. The author also reports that acute synthesis of cytokines in vivo may stimulate and promote the growth of cancer cells and that certain cancer cells may secrete autonomous TNF-α, IL-1 and IL-6. [Id.]

SUMMARY OF THE INVENTION

The invention provides a method of detecting and quantitating the effect of an anti-cancer agent on cancer cells by providing a predetermined number of cancer cells in a cell suspension and culturing the cancer cells in the presence of an anti-cancer agent in cell culture medium that is free of heterologous serum. ATP from the cultured cells is then extracted and stabilized, and then measured in a luminescence reaction.

The invention also provides a method of detecting and quantitating the effect of a combination of anti-cancer agents on cancer cells by providing a predetermined number of cancer cells in a cell

suspension and culturing the cancer cells in the presence of more than one anti-cancer agent in cell culture medium that is free of heterologous serum. The anti-cancer agents may be added to the culture simultaneously or sequentially. ATP from the cultured cells is then extracted and stabilized, and then measured in a luminescence reaction.

The invention further provides kits for performing in vitro assays to detect and quantitate the effect of an anti-cancer agent, or a combination of anti-cancer agents, on cancer cells. The kits generally include containers that comprise media and reagents for performing the methods of the invention.

The methods and kits of the invention allow for more sensitive and accurate detection of anti-cancer agent activity on mammalian cancer cells using bioluminescence and chemiluminescence techniques. In many instances, it is difficult to obtain large numbers of cells from a cancer patient. The invention is advantageous in this respect since it allows for testing of relatively few numbers of cancer cells. Further, since the assays require a fewer number of cells, a greater number of different anti-cancer agents may be tested on the cells and may be tested at multiple concentrations to obtain dose-response data.

In addition, the invention utilizes heterologous serum-free culture medium and culture vessels which prohibit cell attachment, thereby selectively supporting the growth of the cancer cells but not the normal cells that may be present in the culture. Moreover, quantitative results of the assay can be obtained in a relatively short period of time, without the need for expensive or hazardous reagents such as radioisotopes.

Further embodiments and advantages of the present invention will be apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing an ATP standard curve. Methods for generating such a standard curve are described in Example 1 of the application.

Figure 2 is a graph illustrating a comparison of the effects of CDDP, MMC, 5-FU, IFN Alpha 2b, ADM and VP-16 on human colon cancer cells after 6 days of culture in heterologous serum-free medium.

Figure 3 is a graph illustrating a comparison of the effects of CBDCA, ADM, CDDP, 4-HC, CDDP plus 4-HC, and CDDP plus ADM on human ovarian ascites cells after 6 days of culture in heterologous serum-free medium.

Figure 4 is a graph illustrating a comparison of the effects of VP-16, MTX, 5-FU, and the combination treatments of 4-HC, VP-16, plus CBDCA; 4-HC, VP-16, plus CDDP; and MTX, 5-FU, plus CBDCA on human ovarian ascites cells after 6 days of culture in heterologous serum-free medium.

Figure 5 is a graph illustrating a comparison of the effects of ADM, 4-HC, DTIC, CDDP, 5-FU, and the combination treatment of ADM, 4-HC, plus DTIC on human angiosarcoma cells after 6 days of culture in heterologous serum-free medium.

Figure 6 is a graph illustrating a comparison of the effects of ADM, Ara-C, Nov, Prednisone, and the combination treatments of ADM plus Ara-C; and Nov plus Ara-C on human lymphoblastic leukemia cells after 6 days of culture in culture medium containing 10% autologous plasma.

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS OF THE INVENTION

According to the methods of the present invention, mammalian cancer cells are cultured in vitro in the presence of an anti-cancer agent or a combination of anti-cancer agents. The cultured cancer

cells are then tested for sensitivity and resistance to the anti-cancer agent(s) by measuring the luminescence of ATP extracted from the cells. The reference to "cancer cells" in the present invention is used in a broad sense and refers to cells which are usually characterized by unregulated cell growth.

The cancer cells used in the methods of the invention may be obtained from cultured mammalian cell lines. The cell lines may be established from cancerous cells and tissues using standard tissue culture techniques known in the art. Cultured mammalian cancer cell lines can also be obtained from various commercial vendors such as BioWhitaker (Walkersville, Maryland) and from public cell depositories such as the American Type Culture Collection (Rockville, Maryland).

Alternatively, the cancer cells may be obtained directly from a mammal. For instance, the cells can be obtained from a solid tumor, a metastatic tumor, bone marrow, peripheral blood, peritoneal ascites or pleural fluids, as well as other cancerous tissues. The cells can be obtained through various techniques known in the art, including but not limited to, surgical excision or needle biopsy, needle aspiration, venipuncture, thoracentesis, and paracentesis. Preferably, the cells are obtained under relatively aseptic or sterile conditions.

The cancer cells, or tumor specimen containing cancer cells, are placed in an aqueous isotonic or buffered medium. Preferably, the cancer cells are placed in a sterile tissue culture medium. Suitable tissue culture medias are well known to persons skilled in the art and include, but are not limited to, Basal Medium Eagle ("BME"), Minimal Essential Medium ("MEM"), RPMI-1640, Dulbecco's Modified Eagle's Medium ("DMEM"), and McCoy's 5A Medium. These tissue culture medias may be purchased

commercially from various sources, including Sigma Chemical Company (St. Louis, MO) and GIBCO (Grand Island, NY).

More preferably, the cancer cells are placed in a sterile tissue culture medium containing a sufficient amount of antibiotic agent to prevent microbial contamination. Even more preferably, the cancer cells are placed in a sterile tissue culture medium containing penicillin, streptomycin, and gentamicin. Most preferably, the sterile tissue culture medium contains 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml gentamicin. Penicillin, streptomycin, and gentamicin, as well as other tissue culture grade antibiotics known in the art, are commercially available from Sigma and GIBCO, and may be added to the aqueous medium in concentrations recommended by the manufacturer. Microbial contamination may also be reduced by the use of routine sterile techniques in handling the cancer cells and the use of a laminar flow hood.

Optionally, an anti-fungal agent may be added to the aqueous medium to reduce fungal contamination. For example, the anti-fungal agent, amphotericin B (20 μg/ml) (commercially available from Sigma), may be added to the medium. For cancer cells or tumor specimens containing fluids or blood, especially those cancer cells or tumor specimens obtained directly from peripheral blood or pleural fluid, an anti-clotting agent is preferably added to the medium to reduce clotting. Suitable anti-clotting agents are known in the art. A preferred anti-clotting agent is heparin. A preferred concentration of heparin in the medium is about 25 units/ml to about 50 units/ml. The cancer cells are then incubated in the desired aqueous medium. Preferably, the cells are incubated in the medium for several hours at room temperature or overnight at 4°C.

If the cancer cells are obtained from a solid tumor or lymph nodes, the tumor specimen is minced or fragmented into relatively small pieces. The specimen is preferably minced or fragmented using a sterile surgical scalpel. The minced or fragmented specimen is then dissociated to form a cell suspension. Dissociation is preferably accomplished by incubating the minced specimen with an enzymatic agent. It will be readily apparent to those persons skilled in the art that certain types of tumors and cancer cells may have variable susceptibility to enzymatic dissociation. Appropriate types and concentrations of enzymatic agents may be determined empirically, and making such determinations is within the skill in the art without undue experimentation. Applicants have found that an enzymatic agent comprising DNAase and collagenase is preferable to dissociate the cancer cells. In a more preferred embodiment, the enzymatic agent comprises DNAase I (1500 units/ml) and collagenase (2-7 mg/ml), diluted in a tissue culture medium. Preferably, the enzymatic agent is sterile filtered prior to use.

The minced tumor specimen is then incubated with the enzymatic agent for a time sufficient to cause dissociation. It will be readily apparent to those persons skilled in the art that the length of incubation will vary depending on the specimen and the enzymatic agent being employed. Dissociation of the specimen to form a cell suspension may be monitored by visual observation. Preferably, the specimen and enzymatic agent are incubated at 37°C for about 2 to about 20 hours. More preferably, the specimen and enzymatic agent are incubated horizontally in a tube to disperse the specimen along the side of the tube. The tube is then preferably inverted 5-10 times at 30-45 minute intervals to mix the cells. Following incubation, the dissociated specimen is washed and resuspended in fresh medium. In a most preferred

embodiment, relatively large cell aggregates remaining after dissociation are removed from the specimen sample. It is believed, although not fully understood, that relatively large cell aggregates in the specimen may cause undue variability in the methods of the invention resulting from uneven cell distribution during culture. Thus, a cell suspension comprising single cells or aggregates having less than about 50 cells per aggregate is most preferred for culturing. Typically, cancer cells obtained from peripheral blood, peritoneal or pleural fluids, or cultured cell lines do not require dissociation with an enzymatic agent.

The cancer cells are then preferably counted to determine the approximate concentration and viability of the cells. Concentration and viability of the cells may be determined by standard techniques known in the art. For example, cell viability may be determined by trypan blue exclusion methods. It is believed that cell viability should be greater than 60% to ensure more accurate results in the method.

In an optional step, normal cells that may be present in the cancer cell sample may be removed. As used in the present application, the term "normal cells" refers to cells which are not cancer cells. Typically, normal cells such as erythrocytes, as well as dead cells, will be present in the cancer cell sample, especially when the cancer cells are obtained from blood, bone marrow, or peritoneal or pleural fluids. Removal of various normal cells and dead cells in the sample may be accomplished using methods known to persons skilled in the art, such as centrifugation and density gradient separation. Preferably, erythrocytes and dead cells are removed using a ficoll-hypaque gradient separation technique. This technique is further described in Example 1. Ficoll-hypaque and ficoll-hypaque-type products are commercially available and may be used according to the manufacturer's

instructions. Two such products are marketed under the tradenames Histopaque® (Sigma) and Isolymph® (Gallard-Schlesinger, Corte Place, NY). Although not required in the methods of the present invention, it is believed that centrifugation or density gradient separation is useful in reducing the number of dead cells and erythrocytes in the cancer cell sample. Thus, this step may be used to enrich the viability of the cell sample if it is determined to be less than 40%. Following removal of the normal cells, the cancer cell sample is preferably washed and resuspended in tissue culture medium.

The cancer cells are then cultured in vitro in a suitable cell culture medium under conditions sufficient for the cells to remain viable and grow. The cell culture medium employed in the present invention is free of heterologous serum. "Heterologous serum" is defined herein as serum from a source other than the mammal from which the cancer cells were obtained or derived. Although not fully understood, it is believed that certain exogenous growth factors present in heterologous serum may overstimulate cancer cell growth, resulting in a higher sensitivity to the anti-cancer agent(s) being tested in vitro as compared to the in vivo physiological condition. Further, it is believed that the absence of heterologous serum leads to more reproducible results in the methods and kits of the present invention since it is extremely difficult, if not impossible, to utilize identical lots of serum for any extended period of time. Also, the absence of heterologous serum in the cell culture medium aids in preventing growth of normal cells that may be present in the specimen. The cell culture medium should also be free of components which competitively inhibit luminescence reactions and/or anti-cancer agents utilized in the methods of the invention, including ATP, thymidine and reduced folic acid.

Preferably, the cell culture medium comprises a sterile tissue culture medium supplemented with a sufficient quantity of nutrient components. Suitable tissue culture medias include, but are not limited to, BME, MEM, RPMI-1640, DMEM, and McCoy's 5A medium, all of which are commercially available from Sigma or Gibco. Nutrient components contemplated by the invention include, but are not limited to, amino acids, lipids and hormones. The cell culture medium also preferably includes at least one antibiotic agent to prevent microbial contamination. More preferably, the cell culture medium comprises RPMI-1640 or DMEM tissue culture medium, penicillin/streptomycin, amino acids, lipids, and hormones.

In an even more preferred embodiment, the cell culture medium comprises the tissue culture medium and nutrient components shown in Table 1 below.

TABLE 1

Component	Concentration
RPMI-1640 or DMEM (Sigma)	
Hepes Buffer pH 7.2 (Sigma)	25 mM
Kanamycin Sulfate (Gibco)	100 μg/ml
Penicillin/Streptomycin (Gibco)	50 U/ml penicillin 50 μg/ml streptomycin
Sodium Pyruvate (Sigma)	1:100
Trace Element Mix (Gibco)	1:100
Lipids-Cholesterol Rich (Sigma)	0.5%
Albumin Bovine Fraction V (Sigma)	2.0 mg/ml-8.0 mg/ml
Insulin-Transferrin-Selenium (Boerhinger Mannheim)	0.5 mg/ml
Progesterone (Sigma)	5 ng/ml
β-estrodiol (Sigma)	5 ng/ml

In an alternative embodiment, the cell culture medium may comprise a quantity of autologous serum or plasma. "Autologous serum or plasma" is defined herein as a serum or plasma from the mammal from which the cancer cells were obtained or derived. Preferably, the concentration of autologous serum or plasma is about 1% to about 20% of the cell culture medium. Although not completely understood, it is believed that the presence of autologous serum or plasma in the cell culture medium may provide a physiological environment or conditions which are patient specific for growth factors and the like.

The cancer cells are cultured in a vessel suitable for sterile tissue culture, including but not limited to, plates, tubes, and dishes. Preferably, the volume of the vessel is relatively small so that large numbers of cancer cells are not required. The culture vessel may be formed from a variety of materials such as glass or plastic. If ATP luminescence is to be measured directly from the culture vessel, however, the vessel should be relatively transparent or opaque.

More preferably, the cancer cells are cultured in a plate containing a plurality of wells. Multiwell plates contemplated by the invention include 24, 48, and 96 well plates. Multiwell culture plates are commercially available from COSTAR (Cambridge, MA) and Corning (Corning, NY.) Typically, these plates are sold in pre-sterilized form. In a most preferred embodiment, the cancer cells are cultured in round-bottom 96 well culture plates. The optimum number of cancer cells cultured in the culture vessel may be determined empirically by those persons skilled in the art without undue experimentation. In a preferred embodiment, about 1.0×10^4 cells/well to about 4.0×10^4 cells/well are cultured in a 96-well plate.

The culture vessel is selected so that normal cells that may be present in the cancer cell sample are prevented from attaching or adhering to the surfaces of the vessel. Normal cells are typically anchorage-dependent cells, and require adherence or attachment to a solid surface in order to divide and grow. By selecting culture vessels in which these cells cannot anchor or attach, proliferation of the normal cells is prevented but the cancer cells (non-anchorage dependent) are allowed to grow and proliferate.

Attachment of normal cells to the culture vessel may be prevented by coating the inside surfaces of the culture vessel with a material such as agarose. The term "agarose" is used herein in its broadest

sense, and includes relatively pure agarose as well as materials containing agarose, including the compound known as agar. Many agars and agaroses are commercially available, for example, SEAPLAQUE agar (FMC Corporation).

In one embodiment, the inside surfaces of a culture plate are coated with a thin film or layer of agarose. For culturing purposes, both the plate and agarose coating should be sterile. Agarose solutions may be sterilized by heating to relatively high temperatures. Preferably, the agarose solution is sterilized by autoclaving for 15 minutes (250°F at 15 psi). Upon heating, the agarose will go into suspension. Upon cooling, the solution will become a semi-solid gel.

To coat the inside surfaces of the culture plate, the agarose is preferably placed in the plate or in the wells of the plate so that the agarose fills the well and is in contact with all inside surfaces. Then, the agarose is maintained in contact with the surfaces for a time period sufficient for a thin film of agarose to adhere to the surfaces, but before the agarose has gelled or thickened, usually about 5 minutes. The agarose is then shaken out of the wells and off the plate so that a thin, agarose film coats all surfaces of the plate. This can be accomplished by inverting the plate and shaking, which can be done manually or by automation. Preferably, the culture plate is coated with an aqueous agarose solution containing about 0.1% to about 2.0% agarose. The coating is preferably about 0.05 mm to about 0.5 mm in thickness. This thin coating remains on the culture plate surfaces throughout the culture and is advantageous because it reduces drying out, cracking, or shrinking upon storage. Further, the thin coating presents minimal interference when removing the cultured cancer cells

from the culture plates or when extracting ATP from the cells for luminometry.

Alternatively, the cancer cells may be cultured in a polypropylene culture vessel. Polypropylene culture vessels are preferred vessels to prevent attachment of normal cells to the surface of the vessel. In a most preferred embodiment, the cells are cultured in a polypropylene round-bottom 96 well plate.

The cancer cells are cultured in the culture vessel in the presence of an agent which is believed to have anti-cancer activity. Such an agent is referred to herein as an "anti-cancer agent." The anti-cancer agent may be a substance which is naturally-occurring, synthesized or produced by genetic engineering techniques. Anti-cancer agents contemplated by the present invention include, but are not limited to, cytotoxic agents or chemotherapy drugs such as Adriamycin ("ADM"), Mitomycin C ("MMC"), 5-Fluorouracil ("5-FU"), Cisplatin ("CDDP"), Vinblastine ("VBL"), etoposide ("VP-16"), carboplatin ("CBDCA"), methotrexate ("MTX"), dacarbazine ("DTIC"), cytosine arabinoside ("Ara-C"), mitoxantrone ("Nov"), and 4-hydroxycyclophosphamide ("4-HC"). The anti-cancer agents may also include immunoadjuvants, steroids, hormones and cytokines such as Tumor Necrosis Factor. Such anti-cancer agents can be purchased from vendors like Bristol-Myers Squibb Oncology Division (Evansville, IN) (e.g., Cisplatin); Roche (Nutley, NJ) (e.g., 5-FU); Burroughs Wellcome (Research Triangle Park, NC) (e.g., Melphalan); Upjohn (Kalamazoo, MI) (e.g., Prednisone); and Eli Lilly (Indianapolis, IN) (e.g., Vinblastine).

The cells may be cultured in the presence of a single anti-cancer agent or a combination of anti-cancer agents. When the cells are cultured in the presence of a combination of anti-cancer agents, the

agents may be added to the culture simultaneously or sequentially. For some types of cancer cells, combinations of anti-agents may have additive or synergistic effects. For example, as shown in Example 3 of the specification, an additive effect of ADM, 4-HC, and DTIC was observed on cultured human angiosarcoma cells.

The preparation of the anti-cancer agent may be according to manufacturer's instructions. The anti-cancer agent is usually diluted in cell culture medium to the desired concentration. The concentration of the anti-cancer agent tested in the cell culture may vary depending on the type of agent and the cancer cells, the kind of cell culture medium employed, and the length of time in the cell culturing period. The desired concentrations may be determined empirically and it is within the skill in the art to make such determinations. Preferably, the anti-cancer agent is tested in duplicate or triplicate wells and more preferably, the anti-cancer agent is tested at several different concentrations in the cell culture so that dose response data can be obtained.

The anti-cancer agent concentrations may be selected using what are known in the art as "PPC" values. A PPC value is an estimated peak plasma concentration for a standard intravenous therapeutic dose of the anti-cancer agent *in vivo*. The PPC value for each anti-cancer agent may be determined empirically, and it is within the skill of those persons skilled in the art to determine the PPC value. Some PPC values are also published. [*See, e.g.*, Alberts et al., *Cloning of Human Tumor Cells*, Alan R. Liss, Inc., New York (1980), pp. 351-359; Dorr et al., *Cancer Chemotherapy Handbook*, Elsevier, New York (1980)]. An example of estimated PPC values for several anti-cancer agents is shown in Table 2 below. Various dilutions of PPC concentrations (for instance, 200, 100, 50, 25,

12.5, 6.25, and 3.13 PPC%) of the agent may be tested in triplicate in 96 well plates.

TABLE 2

Anti-Cancer Agent	PPC µg/ml	In Vivo Drug Dose Correlation
Actinomycin D (Dactinomycin)	0.1	I.V. 500 µg/m^2
Bleomycin (Blenoxane)	3.0	I.V. 15 U/m^2
Carboplatin (Paraplatin)	15.8	I.V. 300 mg/m^2
Carmustine (BCNU, BiCNU)	2.0	I.V. 95 mg/m^2
Cisplatin (Platinol)	3.8	I.V. 100 mg/m^2
Cytosine Arabinoside (Ara-C, Cytosar, Cytarabine)	2.4	I.V. 100 mg/m^2
Dacarbazine (DTIC)	10.0	I.V. 150 mg/m^2
Daunorubicin (Daunomycin)	0.4	I.V. 100 mg/m^2
Doxorubicin (Adriamycin)	0.5	I.V. 60 mg/m^2
Epirubicin	0.5	I.V. 75 mg/m^2
Etoposide (VePesid, VP-16)	48.0	I.V. 100 mg/m^2
5-Fluorouracil (5-FU)	22.5	I.V. 500 mg/m^2
4-HC (Cyclophosphamide, Cytoxan)	3.0	I.V. 1000 mg/m^2
Interferon Alpha-2b	200 IU/ml	I.V. 5,000,000 IU/m^2
Leucovorin	1.2	I.V. 25 mg/m^2
Levamisole	0.5	P.O. 150 mg
Methotrexate (MTX)	2.8	I.V. 25 mg/m^2
Mitoxantrone (Novantrone)	0.3	I.V. 15 mg/m^2
Mitomycin C (Mutamycin)	0.2	I.V. 10 mg/m^2
PALA	18.0	I.V. 150 mg/m^2
Prednisone	62.0	40 mg/m^2 P.O.
Streptozocin (Zanosar)	19.0	I.V. 200 mg/m^2
Taxol	6.8	I.V. 275 mg/m^2
6-Mercaptopurin	25.0	50 mg/m^2 P.O.
6-Thioguanine	9.2	100 mg/m^2 P.O.
Thio-Tepa	1.0	I.V. 12 mg/m^2
Tamoxifen	0.5	P.O. 40 mg
Vinblastine (Velban)	0.5	I.V. 6.0 mg/m^2
Vincristine (Oncovin)	0.4	I.V. 1.5 mg/m^2
Vindesine (Eldisine)	0.6	I.V. 1.5 mg/m^2

For comparative purposes, appropriate controls should also be cultured and tested to determine the efficacy of the anti-cancer agent(s) and to quantitate results. For instance, controls which comprise a quantity of cancer cells but no anti-cancer agent, or which comprise cancer cells and a growth inhibitor should be cultured and tested. These appropriate controls are described further in Example 1 below. The effects of the anti-cancer agents can be determined or calculated by comparison with such control cultures comprising untreated and/or completely killed cancer cells.

The culture vessel containing the cancer cells and anti-cancer agent(s) (or controls) is then incubated under conditions sufficient for the cells to remain viable and grow. Preferably, the culture vessels are incubated in a humidified chamber for about 4 to about 7 days. If rapid cell proliferation is observed, however, the culture duration may be reduced to about 3 days. The specific temperature and time of incubation, as well as other culture conditions, can be varied depending on such factors as the concentration of the anti-cancer agent, the type of cancer cells, and the like. Those skilled in the art will be able to determine operative and optimal culture conditions without undue experimentation.

After the desired culture period, ATP from the cultured cancer cells is extracted and stabilized. As used in the present invention, the term "ATP" refers to adenosine triphosphate. The ATP is extracted from the cultured cells using an extraction reagent. Preferably, the extraction agent comprises an effective amount of ammonium vanadate. The ammonium vanadate should be added in proportions sufficient to effectively inhibit adverse ATPase enzymes that may be present in the sample. More preferably, the extraction reagent comprises about 8 mM to about 14 mM ammonium

vanadate. The presence of ammonium vanadate in the extraction reagent is believed to stabilize the ATP extracted from the cultured cells. It is also believed that reproducible results can be obtained on the extracted ATP for up to about four hours after the ATP extraction step. Stabilizing the extracted ATP also allows time for measuring ATP from a multitude of cultured samples.

In a preferred embodiment, ATP is extracted from the cultured cells using an extraction reagent comprising NH_4VO_3 (ammonium meta vanadate), buffer, and a surface active agent. Preferably, the ammonium vanadate is about 8 mM to about 14 mM of the extraction reagent. Ammonium vanadate is commercially available from various sources, including Sigma.

The buffer included in the extraction reagent is a buffer which adjusts pH to about 7.7 to about 7.9. More preferably, the buffer is one which adjusts pH to about 7.8, which is the optimum pH for the firefly bioluminescence reaction. The buffer is most preferably Hepes buffer. Hepes buffer is commercially available from Sigma. Preferably, the extraction reagent comprises about 0.1 M to about 0.34 M Hepes.

The extraction reagent also preferably comprises a surface active agent. The term "surface active agent" refers generally to a bipolar molecule having a hydrophilic group and a hydrophobic group. Typically, the surface active agent acts as a detergent to solubilize cell membranes. Preferably, the surface active agent in the extraction reagent is a non-ionic detergent such as NP-40 or Triton-X 100. More preferably, the NP-40 or Triton X-100 comprises about 0.03% to about 0.50% by weight of the extraction reagent. Most preferably, the NP-40 or Triton X-100 comprises about 0.10% to about 0.20% by weight of the extraction reagent. NP-40 and Triton X-100 are both commercially available from Sigma.

The extraction reagent may be added directly to the culture vessel and mixed with the cultured cells. The extraction agent is added to the cultured cells so that it comprises about 20% to about 33% of the aqueous medium in the culture vessel. More preferably, the extraction agent is added to the cultured cells in the vessel so that the concentration of ammonium vanadate is about 1.6 mM to about 4.6 mM and the concentration of the surface active agent is about 0.02% to about 0.07%. The cells and extraction agent are then allowed to incubate for about 15 to about 30 minutes. Applicants have found that superior results are achieved when the incubation period does not exceed 60 minutes. Preferably, the cells and extraction reagent are incubated at room temperature.

Next, to measure the ATP, aliquots are removed from each test sample in the culture vessel and mixed with a counting reagent. Preferably, about 0.025 ml to about 0.10 ml aliquots of each sample are transferred to a counting vessel. The counting vessel is an opaque or transparent tube, vial or plate. Then, a sufficient quantity of counting reagent is added to the counting vessel containing the test sample so that results can be measured in about 1 to about 10 seconds. Preferably, about 0.025 ml to about 0.1 ml of counting reagent is added to the counting vessel containing the test sample. Most preferably, the counting reagent is added in excess of the quantity of test sample in the counting vessel. The counting reagent comprises luciferin and luciferase, both of which are commercially available from Sigma and R&D Systems Inc. (Minneapolis, MN). The counting reagent preferably comprises firefly luciferase, D-luciferin, $MgSO_4$, and buffer. In a more preferred embodiment, the counting reagent comprises about 2.0 to about 5.0 µg/ml firefly luciferase (EC 1.13.12.7 from Photinus Pyralis) and about 0.2 mM D-luciferin, diluted in a pH 7.8 buffer

comprising 4.76 mg/ml Hepes and 1.22 mg/ml magnesium sulfate. Preferably, the counting reagent is prepared and incubated 30 minutes prior to use. Because counting reagents comprising luciferin and luciferase are light sensitive, the reagents should be protected from direct light exposure.

The luminescent counts are then measured over a 1-20 second period in a luminometer, or other object capable of measuring light emission and intensity. The luminometer measures the light generated by the ATP extracted from the cultured cancer cells when that ATP is converted in the luciferin-luciferase reaction. Luminometers are known in the art and are commercially available. Suitable luminometers for use in the present invention include the Berthold LB-953 tube luminometer and the Berthold LB-96P microplate luminometer. The luminescent counts are preferably performed 2-4 seconds after adding the counting reagent to the counting vessel. It is also recommended to count an ATP standard and generate an ATP standard curve for luminometry quality control. A preferred ATP standard curve for the methods of the invention is generated using serial 1:3 dilutions of 250 ng/ml ATP in Hepes buffer (pH 7.8) containing 1% bovine serum albumin. An ATP standard curve generated using such serial dilutions is shown in Figure 1. The preparation and counting of ATP standards is further described in Example 1 below.

Alternatively, the extracted ATP from the cells may be stored frozen for luminometer counting at a later date. To freeze the samples, the culture vessels containing the samples should be covered and then frozen below -4°C. Before adding the counting reagent and luminometer counting, the vessels containing the samples should be thawed at 37°C.

It should be kept in mind that any of the steps of the methods may be performed manually or by automation, or a combination thereof.

The present invention also provides a kit for performing in vitro assays to detect and quantitate the effects of anti-cancer agents on cancer cells. The kit is a packaged combination of one or more containers holding compositions or reagents useful in performing the methods of the invention. Many of these compositions and reagents have been described more fully above in relation to the methods of the invention. Suitable containers for the compositions or reagents include, for example, bottles, vials, test tubes, and plates. The containers may be formed from a variety of materials such as glass or plastic.

The kit will generally comprise one or more containers holding enzymatic reagent for dissociating cancer cells or tumor specimens containing cancer cells, ATP extraction reagent, and heterologous serum-free cell culture medium. The kit may also comprise one or more containers holding luminescence counting reagent and ATP standard or inhibitor. The compositions or reagents in the kit may be in solution or in a lyophilized form.

The kit may further comprise other materials which are known in the art and which may be desirable from a commercial and user standpoint such as buffers, diluents, filters, scalpels, etc. The kit may also include containers like test tubes, vials, or culture plates such as multiwell plates for culturing the cancer cells.

In a most preferred embodiment, the kit comprises the following: at least one sterile scalpel; at least one 10 ml syringe; at least one 0.22 μm filter unit; at least one sterile polypropylene 96-well culture plate; and containers, separately packaged, that include the following: 250 ml heterologous serum-

free cell culture medium; lyophilized enzymatic reagent [7.5 mg DNAase Type I; 70 mg Collagenase Type H]; 2 ml maximum ATP inhibitor [3.125 mM Hepes pH 7.2, 0.0125% Triton X-100, diluted in RPMI-1640 medium]; 20 ml ATP extraction reagent [1.2672 mg/ml ammonium vanadate; 0.5% Triton X-100; 0.1017 g/ml Hepes pH 7.8]; lyophilized counting reagent [2.0 - 5.0 μg/ml luciferase, 0.2 mM luciferin]; lyophilized ATP standard [1.0 mg bovine serum albumin; 500 ng ATP]; and 20 ml dilution buffer [4.76 mg/ml Hepes pH 7.8, 1.22 mg/ml magnesium sulfate].

EXAMPLES

In vitro assays were performed to detect and quantitate the sensitivity and/or resistance of human cancer cells to different anti-cancer agents. The sensitivity and/or resistance was evaluated by measuring luminescence of ATP from the cultured cells using a luciferin-luciferase reaction.

Example 1: Effects of Anti-Cancer Agents on Human Colon Cancer Cells

A. Solid Tumor Specimen Preparation

A colon tumor biopsy specimen was surgically obtained from patient "SD" diagnosed with colon cancer (Mount Sinai Medical Center, NY). The tumor specimen was placed immediately in sterile, DMEM medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml kanamycin. The specimen was processed under aseptic conditions, transported on ice to maintain cell viability and avoid microbial contamination, and stored at 2 - 8°C.

The specimen was then placed in a 100 x 15 mm sterile petri dish. Excess fat and connective tissue was excised from the specimen using sterile scalpels and discarded. The specimen was then minced into approximately 0.5 - 2.0 mm^3 pieces using the sterile

scalpels. Next, approximately 0.5 - 1.0 gram (0.5 - 1.0 ml) of the minced specimen was transferred into a sterile 15 ml conical centrifuge tube containing approximately 10 ml of enzymatic reagent [0.75 mg/ml DNAase Type I (Sigma); 7.0 mg/ml Collagenase Type H (Sigma)] which had been sterile filtered using a 10 ml syringe and a 0.22 μm filter (Gelman Sciences Acrodisk filter).

The tumor specimen was dissociated by incubating and mixing the minced tissue in the enzymatic reagent for about 4-6 hours at 37°C. The specimen was incubated horizontally in the conical centrifuge tube to disperse the minced tissue along the side of the tube. The specimen was further disrupted by inverting the tube and mixing the cells 5-10 times at 30-45 minute intervals until a dissociated cell suspension was evident.

The tube containing the dissociated cell suspension was then centrifuged at 400 x g for 5-8 minutes, and the supernatant was discarded. The pelleted specimen was washed twice in 10 ml of cell culture medium [as described in Table 1, using DMEM medium] by centrifugation at 400 x g for 5-8 minutes. Following centrifugation, the specimen was resuspended in fresh cell culture medium.

The resuspended specimen was then incubated vertically in the conical centrifuge tube for 5-10 minutes to allow the non-dissociated tumor aggregates to settle out of solution. The cell culture medium supernatant containing the dissociated tumor cell suspension was transferred to another sterile 15 ml conical centrifuge tube using a sterile 10 ml pipette, and the non-dissociated tumor aggregates were discarded. The concentration and viability of the tumor cell suspension was then determined by trypan blue exclusion.

After counting the cells, ficoll-hypaque density gradient separation was performed to reduce dead cell and erythrocyte contamination in the cell suspension. Approximately 5 ml of sterile, room temperature ficoll-hypaque (purchased from Sigma) was added to sterile 15 ml conical centrifuge tubes using a sterile 5 ml pipette. Next, 10 ml of the cell suspension was gently overlayed on the Ficoll-hypaque using a sterile 10 ml pipette. Care was taken to avoid mixing the Ficoll-hypaque and cell suspension layers.

The tubes were then centrifuged at 400 x g for 30 minutes at room temperature, and the upper cell culture medium layer was removed and discarded. The viable cells at the cell culture medium-Ficoll-hypaque interface were then transferred to another sterile 15 ml conical centrifuge tube using a sterile 5 ml pipette. The cells were washed twice with 10 ml of cell culture medium by centrifugation at 400 x g for 5-8 minutes and resuspended in fresh cell culture medium. Cell concentration and viability was again determined by trypan blue exclusion. The washed and counted tumor cells were resuspended in cell culture medium at a concentration of about 2.0×10^5 viable cells/ml.

B. Preparation of Anti-Cancer Agents

Six different agents were tested in the assay: ADM, MMC, 5-FU, CDDP, VP-16, and Interferon Alpha 2b ("IFN Alpha 2b") (purchased from South Florida Health Alliance, Inc.). Each agent was tested at 7 different concentrations corresponding to 200%, 100%, 50.0%, 25.0%, 12.5%, 6.25% and 3.13% of the estimated Peak Plasma Concentration (PPC). The 100% PPC value corresponds to the estimated maximum achievable blood level of the test agent for the in vivo dose as shown in Table 2. The mean of 25% to 3.13% PPC low concentrations was used as an estimate of the in vivo exposure.

First, stock solutions of each agent were prepared and stored according to manufacturer's instructions. A 800% PPC test solution was then prepared for each agent by diluting the respective stock solutions in 10 ml of cell culture medium.

C. Tumor Cell Cultures

The tumor cells were cultured in sterile 96-well polypropylene round bottom plates with L.E. lids (purchased from COSTAR, Cambridge, MA). Triplicate cultures were tested for each concentration of anti-cancer agent. The remaining wells of the 96 well-culture plates were used for "No Drug" and "Maximum Inhibitor" controls.

Using an adjustable pipette, 0.1 ml of Maximum ATP Inhibitor Reagent [3.125 mM, pH 7.2 Hepes buffer (Sigma) and 0.0125% Triton X-100 (Bio-Rad) diluted in DMEM medium (Gibco)] was added to designated wells of the culture plate used for "Maximum Inhibitor" controls. Using a multichannel pipette, 0.1 ml of DMEM cell culture medium was then added to each well of the culture plates used to test the anti-cancer agents, and to wells of the culture plates used for "No Drug" controls.

Using an adjustable pipette, 0.1 ml of the 800% PPC test solution of each anti-cancer agent was added to triplicate wells of the culture plates. Then, 0.1 ml of the anti-cancer agent solutions were serially diluted 1:2 in selected wells of the plate.

Next, 0.1 ml of the tumor cells (2.0×10^5 viable cells/ml DMEM cell culture medium) was added to the Maximum ATP Inhibitor and No Drug control wells of the culture plates. Using a multichannel pipette, 0.1 ml of the tumor cells was then added to the remaining wells in the culture plates containing anti-cancer agent dilutions. Addition of 0.1 ml of cells provided a concentration of 2.0×10^4 viable cells/well in 0.2

ml of DMEM culture medium and diluted the test agent solutions to recommended PPC test concentrations of 200%, 100%, 50.0%, 25.0%, 12.5%, 6.25% and 3.13% in respective wells of the culture plates.

The culture plates were then incubated in a 95% humidified, 37°C, 5% CO_2 incubator for about 6 days.

D. ATP Extraction and Counting

Using a multichannel pipette, 0.075 ml of ATP Extraction Reagent [1.2672 mg/ml ammonium vanadate (Sigma), 0.5% Triton X-100 (Bio-Rad), 0.1017 g/ml Hepes pH 7.8 (Sigma)] was added to the Maximum ATP Inhibitor and No Drug control wells in the culture plates. The cells were then immediately mixed by gently pipetting the cultures 4 times using a multichannel pipette while avoiding bubbling of the culture medium.

Next, 0.075 ml of the ATP Extraction Reagent was added to the remaining wells in the culture plates containing tumor cells and anti-cancer agent. The cells were immediately mixed by gently pipetting the cultures 4 times using a multichannel pipette while avoiding bubbling of the culture medium. The plates were incubated 20 minutes at room temperature.

Samples were then prepared for luminometer counting. Results were obtained when the samples were counted within 60 minutes after addition of the ATP Extraction Reagent. Using an adjustable pipette, 0.05 ml aliquots from each well were transferred to luminometer counting plates, and 0.05 ml of Counting Reagent [2.0-5.0 μg/ml firefly luciferase (Sigma), 0.2 mM D-luciferin (Sigma), diluted in 4.76 mg/ml Hepes pH 7.8, 1.22 mg/ml magnesium sulfate] was added.

Counting of the test samples was performed using a Berthold luminometer, and a count integration time of 10 seconds.

ATP standard counts were also determined. Lyophilized ATP Standard [bovine serum albumin (Sigma) 10 mg (1%); ATP standard (Sigma) (500 ng/vial)] was reconstituted with 2 ml of Dilution Buffer [4.76 mg/ml Hepes pH 7.8, 1.22 mg/ml magnesium sulfate] to give a stock ATP concentration of 250.0 ng/ml. Using an adjustable pipette, 0.30 ml of the Dilution Buffer was added to nine (9) sterile dilution tubes. Using an adjustable pipette, 0.15 ml of the stock ATP Standard was serially diluted through the nine 0.30 ml aliquots of Dilution Buffer to give ATP Standard concentrations of 83.33 (1:3), 27.76 (1:9), 9.253 (1:27), 3.084 (1:81), 1.028 (1:243), 0.342 (1:729), 0.114 (1:2187), 0.038 (1:6561), and 0.012 (1:19683) ng/ml. Triplicate 0.05 ml samples of each ATP Standard dilution were then counted in the Berthold luminometer, along with triplicate 0.05 ml samples of the stock ATP Standard (250.0 ng/ml) and Dilution Buffer (0.000 ng/ml). A count integration time of 10 seconds was utilized. The ATP standard curve obtained is shown in Figure 1.

When counting was completed, the Counting Reagent was removed from the luminometer tubing. The tubing was then rinsed with 70% ethanol in sterile water and left dry between use to avoid microbial contamination.

Log mean luminometer counts were plotted for each ATP Standard concentration vs. log ATP Standard concentration to determine standard curve linearity. A correlation coefficient of $R > 0.975$ was determined by linear regression analysis.

The luminometry results for all test samples were then calculated and interpreted according to the equation and criteria set forth in Table 3 below.

TABLE 3

CALCULATION AND
INTERPRETATION OF LUMINOMETRY RESULTS

The percentage of cancer cell growth inhibition for each concentration of anti-cancer drug or drug combination was calculated by the equation:

$$1.0 - \frac{(TEST)-(MI)}{(MO)-(MI)} \times 100 = \text{Percent Tumor Growth Inhibition}$$

(TEST) = Mean counts for test drug cultures.

(MI) = Mean counts for Maximum Inhibitor Controls.

(MO) = Mean counts for no drug controls.

The interpretation of luminometry results to determine sensitivity and resistance can be based on the mean percent inhibition for high anti-cancer drug concentrations of 200% - 50.0% PPC, and low anti-cancer drug concentrations of 25.0% - 3.13% PPC. The 100% PPC value corresponds to the estimated maximum achievable blood level of the anti-cancer drug for the *in vivo* drug dose correlation shown in Table 2.

In this assay, drug sensitivity and resistance were defined generally according to five dose-response criteria:

STRONG SENSITIVITY - Mean percent inhibition is greater than 70% for both high test concentrations of 200% - 50.0% PPC and low test concentrations of 25.0% - 3.13% PPC. Strong sensitivity is most evident by high levels of inhibition at low test drug concentrations.

INTERMEDIATE SENSITIVITY - Mean percent inhibition is greater than 70% for high test concentrations of 200%-50.0% PPC, and is 50%-70% for low test concentrations of 25.0%-3.13% PPC.

WEAK SENSITIVITY - Mean percent inhibition is greater than 50% for high test concentrations of 200%-50.0% PPC, and is less than 50% for low test concentrations of 25.0%-3.13% PPC.

PARTIAL SENSITIVITY - Mean percent inhibition is 50%-70% for both high test concentrations of 200%-50.0% PPC and low test concentrations of 25.0%-3.13% PPC.

RESISTANCE - Mean percent inhibition is less than 50% for both high test concentrations of 200%-50% PPC and low test concentrations of 25.0%-3.13% PPC. Resistance is most evident by low levels of inhibition at high test agent concentrations.

Using the above criteria, the results of the assay indicated that the colon cancer cells had a strong sensitivity to ADM, intermediate sensitivity to MMC, and weak sensitivity to CDDP, 5-FU, and VP-16. The assay also revealed that the patient's colon cancer

cells were resistant to IFN alpha 2b. These results are illustrated graphically in Figure 2.

Example 2: Effects Of Anti-Cancer Agents On Human Ovarian Ascites Cells

An ovarian ascites sample was obtained by paracentesis from patient "GM" at North Ridge Hospital in Ft. Lauderdale, Florida. The specimen was first placed in DMEM tissue culture medium containing 50 units/ml sodium heparin to prevent clotting. The specimen was then dissociated and the cells were cultured as described in Example 1 above.

The effects of seven single anti-cancer agents (VP-16, CDDP, CBDCA, 4-HC, MTX, ADM, and 5-FU) were tested. Five different combination treatments were also tested: 4-HC, VP-16 plus CBDCA; 4-HC, VP-16 plus CDDP; 5-FU, MTX plus CBDCA; CDDP plus 4-HC; and CDDP plus ADM. The anti-cancer agents were purchased from South Florida Health Alliance, Inc. The cultured cells were tested with 7 concentrations of each single agent, or the combination of agents, equivalent to 200%, 100%, 50%, 25%, 12.5%, 6.25% and 3.13% of the Peak Plasma Concentration (PPC). (See Table 2, 5-FU was tested at PPC = 11.25 mcg/ml). In all anti-cancer agent combination treatments, the agents were added simultaneously to the respective test wells.

After 6 days of culture, ATP from the cultured cells was extracted and stabilized, and counted as described in Example 1. Luminometry counts were then analyzed and interpreted according to the equation and criteria described in Example 1, Table 3.

The results of the assay are shown in Figures 3 and 4. It was found that the cultured ovarian ascites cells were resistant to individual treatments of VP-16, MTX, 4-HC, CDDP, and CBDCA. The cultured cells exhibited weak sensitivity to 5-FU and ADM, and the combination treatments of MTX, 5-FU, plus CBDCA; CDDP plus ADM; and CDDP plus 4-HC. Intermediate

sensitivity was observed for the combination treatments of 4-HC, VP-16 plus CBDCA, and 4-HC, VP-16 plus CDDP. A synergistic effect was observed for the combination treatments of 4-HC, VP-16 plus CBDCA; 4-HC, VP-16 plus CDDP; MTX, 5-FU plus CBDCA; and CDDP plus 4-HC.

Example 3: Effects Of Anti-Cancer Agents On Human Angiosarcoma Cells

An angiosarcoma tumor biopsy was surgically obtained from patient "SW" at North Ridge Hospital in Ft. Lauderdale, Florida. The specimen was dissociated and the cells were cultured as described in Example 1 above. The effects of five single anti-cancer agents (4-HC, ADM, DTIC, CDDP, and 5-FU) and the combination of three agents (ADM, 4-HC, plus DTIC) were tested. The anti-cancer agents were purchased from South Florida Health Alliance, Inc. The cultured cells were tested with 7 concentrations of each single agent, or the combination of agents, equivalent to 200%, 100%, 50%, 25%, 12.5%, 6.25% and 3.13% of the Peak Plasma Concentration (PPC). (See Table 2, 5-FU was tested at PPC = 11.25 mcg/ml). In the combination treatment, ADM, 4-HC and DTIC agents were added simultaneously to the respective test wells.

After 6 days of culture, ATP from the cultured cells was extracted and stabilized, and then counted as described in Example 1. Luminometry counts were then analyzed and interpreted according to the equation and criteria described in Example 1, Table 3.

The results of the assay are shown in Figure 5. It was found that the cultured angiosarcoma cells were resistant to DTIC, CDDP, and 5-FU. Weak sensitivity was observed for cells treated with ADM, and intermediate sensitivity was observed for cells treated with 4-HC. The cultured cells exhibited strong sensitivity to the combination treatment of ADM plus 4-HC plus DTIC. As illustrated in Figure 5, an additive

effect was also observed for the cells treated with a combination of ADM plus 4-HC plus DTIC.

Example 4: Effects Of Anti-Cancer Agents On Human Lymphoblastic Leukemia Cells

A venous blood specimen was obtained from patient "LS" at Parkway Hospital in Miami, Florida. The lymphoblastic leukemia blood sample contained about 70% blast cells. The specimen was first placed in RPMI-1640 tissue culture medium containing 50 units/ml sodium heparin to prevent clotting. The cells were prepared for the assay by ficoll-hypaque density gradient centrifugation as described in Example 1. The sample, however, was not dissociated with enzymatic agent. The cells were cultured as described in Example 1 except that the cell culture medium [described in Table 1, using RPMI-1640 medium] contained 10% autologous plasma.

The effects of four single anti-cancer agents (ADM, Ara-C, Nov, and Prednisone) were tested. Two different combination treatments were also tested: ADM plus Ara-C, and Nov plus Ara-C. The anti-cancer agents were purchased from South Florida Health Alliance, Inc. The cultured cells were tested with 7 concentrations of each single agent, or combination of agents, equivalent to 200%, 100%, 50%, 25%, 12.5%, 6.25%, and 3.13% of the Peak Plasma Concentration (PPC). In the anti-cancer agent combination treatments, the agents were added simultaneously to the respective test wells.

After 6 days of culture, ATP from the cultured cells was extracted and stabilized, and counted as described in Example 1. Luminometry counts were then analyzed and interpreted according to the equation and criteria described in Example 1, Table 3.

The results of the assay are illustrated graphically in Figure 6. The cultured cells showed strong sensitivity for ADM and the combination treatment of ADM plus Ara-C. The cultured cells

exhibited an intermediate sensitivity for Ara-C and the combination treatment of Nov plus Ara-C, and exhibited weak sensitivity to Nov and prednisone treatments.

WHAT IS CLAIMED IS:

1. A method for detecting and quantitating the effect of an anti-cancer agent on cancer cells, comprising the steps of:

providing a predetermined number of cancer cells in a cell suspension;

culturing said cells in the presence of an anti-cancer agent, said cells being cultured in medium free of heterologous serum;

extracting and stabilizing ATP from said cultured cells; and

measuring the luminescence of said ATP as an indicator of the effect of said agent on said cells.

2. The method of claim 1 wherein said cells are cultured in a culture vessel that prohibits attachment of anchorage-dependent cells in the culture.

3. The method of claim 1 whereby ATP from said cells is extracted and stabilized by contacting the cultured cells with a sufficient amount of extraction reagent comprising ammonium vanadate.

4. The method of claim 1 wherein said anti-cancer agent is a chemotherapy drug.

5. The method of claim 1 wherein a quantity of autologous serum is added to the cell culture medium.

6. The method of claim 1 wherein a quantity of autologous plasma is added to the cell culture medium.

7. A method for detecting and quantitating the effect of a combination of anti-cancer agents on cancer cells, comprising the steps of:

providing a predetermined number of cancer cells in a cell suspension;

culturing said cells in the presence of more than one anti-cancer agent, said cells being cultured in medium free of heterologous serum;

extracting and stabilizing ATP from said cultured cells; and

measuring the luminescence of said ATP as an indicator of the effect of said agents on said cells.

8. The method of claim 7 wherein said anti-cancer agents are added to the cell culture sequentially.

9. The method of claim 7 wherein said cells are cultured in a culture vessel that prohibits attachment of anchorage-dependent cells in the culture.

10. The method of claim 7 whereby ATP from said cells is extracted and stabilized by contacting the cultured cells with a sufficient amount of extraction reagent comprising ammonium vanadate.

11. The method of claim 7 wherein said anti-cancer agents are chemotherapy drugs.

12. The method of claim 7 wherein a quantity of autologous serum is added to the cell culture medium.

13. The method of claim 7 wherein a quantity of autologous plasma is added to the cell culture medium.

14. A kit for detecting and quantitating the effects of at least one anti-cancer agent on cancer cells, the kit comprising one or more containers holding:

(a) an enzymatic reagent for dissociating cancer cells;

(b) an extraction reagent for extracting ATP from cancer cells, said extraction reagent comprising ammonium vanadate; and

(c) cell culture medium free of heterologous serum.

15. The kit of claim 14 wherein said cell culture medium comprises the components described in Table 1 of the specification.

16. The kit of claim 14 wherein the kit further comprises:

(d) an ATP luminescence counting reagent;

(e) an ATP standard; and

(f) an ATP inhibitor.

17. The kit of claim 16 wherein the kit further comprises:

(g) at least one sterile scalpel; and

(h) at least one polypropylene 96 well culture plate.

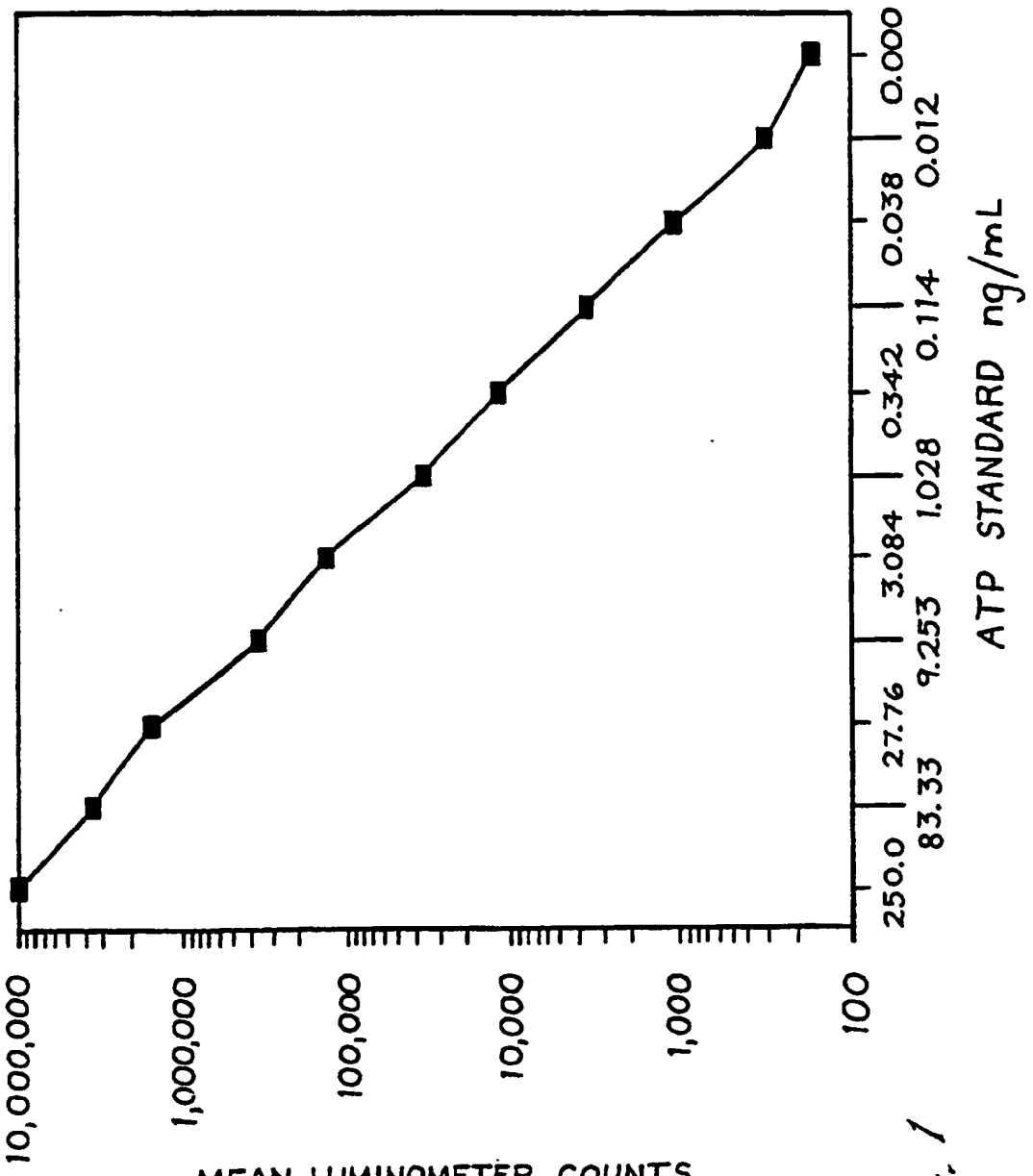

10,000,000
1,000,000
100,000
10,000
1,000
100
MEAN LUMINOMETER COUNTS
250.0
83.33
27.76
9.253
3.084
1.028
0.342
0.114
0.038
0.012
0.000
ATP STANDARD ng/mL

Fig. 1

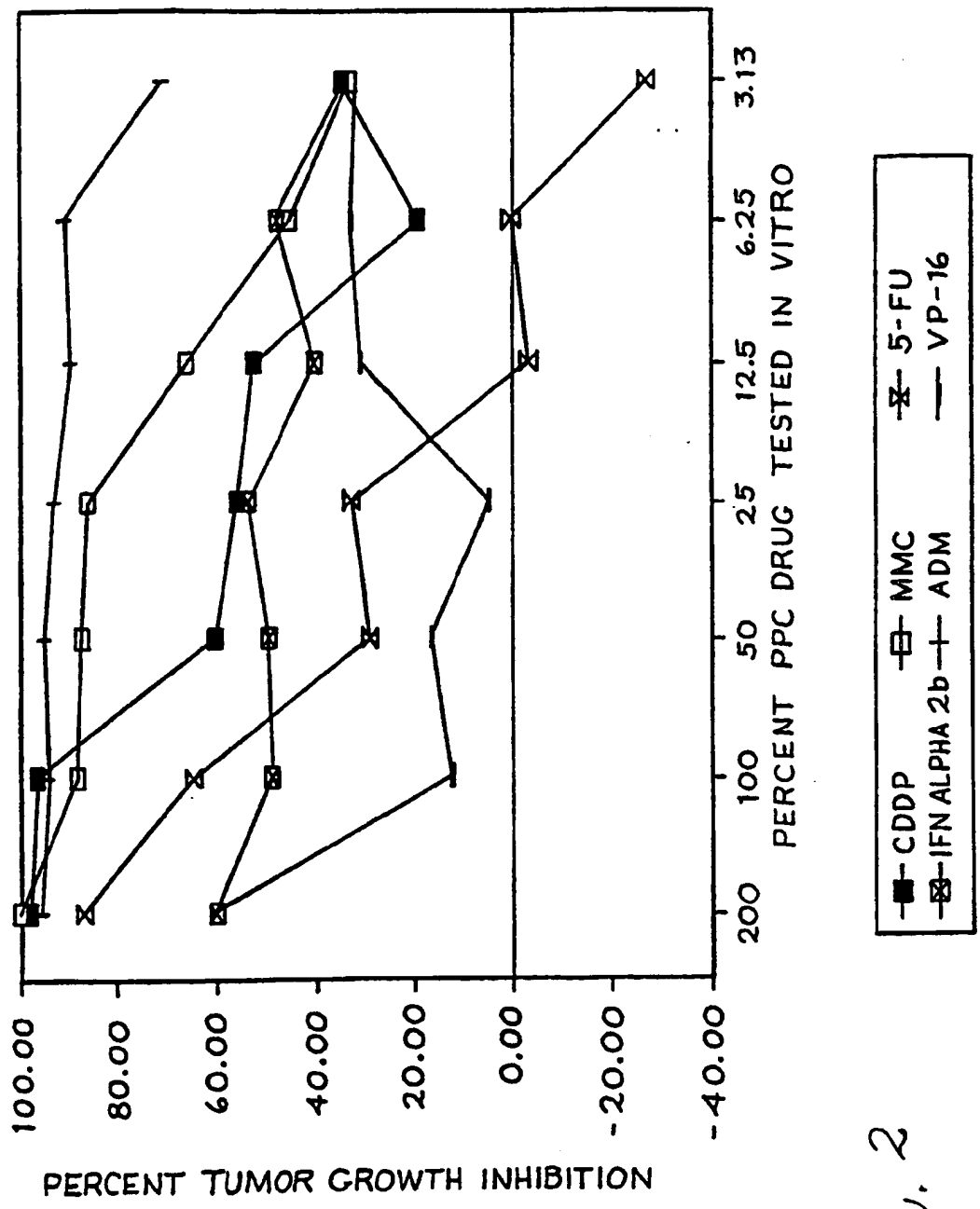

100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
PERCENT TUMOR GROWTH INHIBITION
200
100
50
25
12.5
6.25
3.13
PERCENT PPC DRUG TESTED IN VITRO
CDDP
MMC
5-FU
IFN ALPHA 2b
ADM
VP-16

Fig. 2

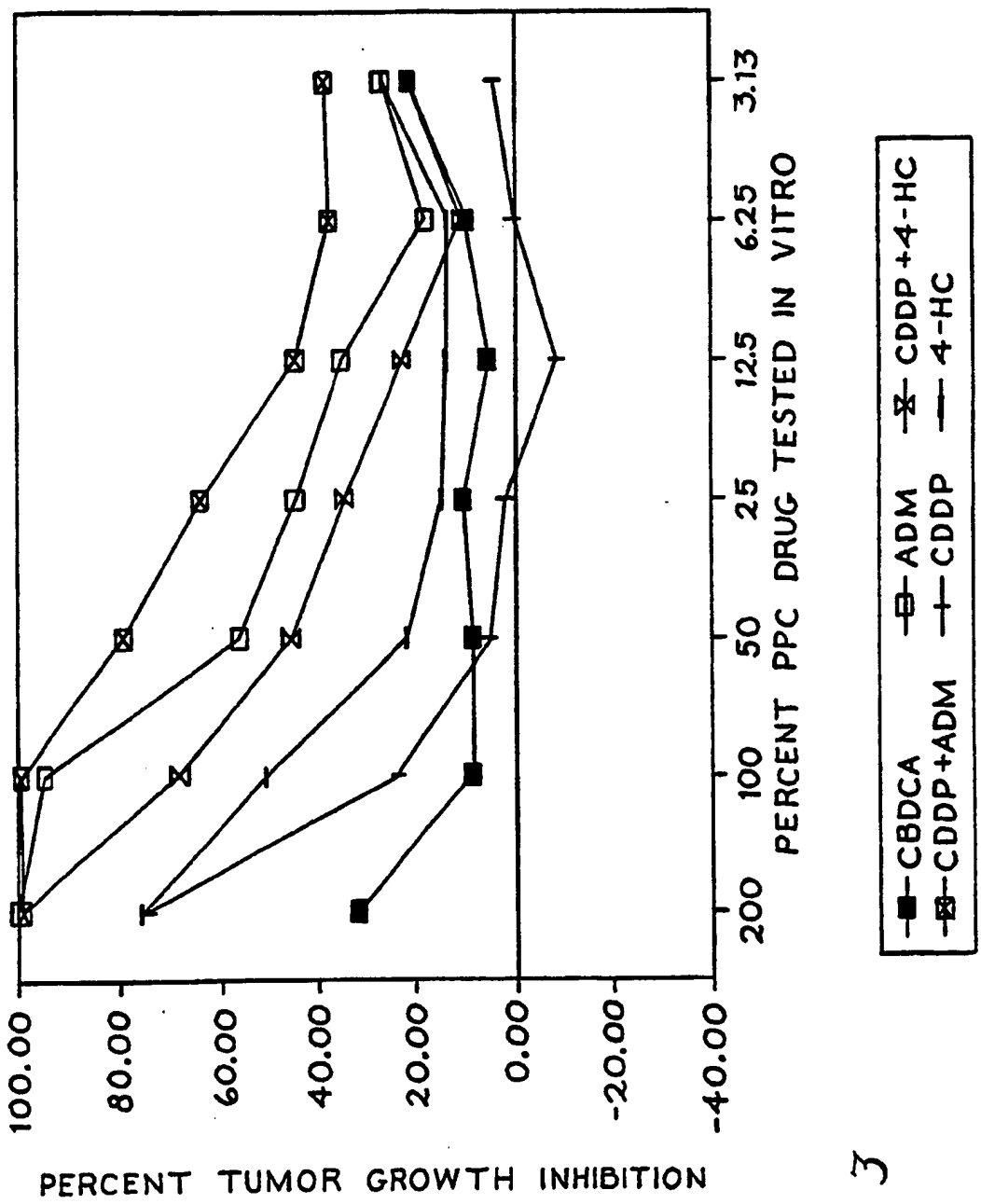

PERCENT TUMOR GROWTH INHIBITION
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
PERCENT PPC DRUG TESTED IN VITRO
200
100
50
25
12.5
6.25
3.13
CBDCA
ADM
CDDP+4-HC
CDDP+ADM
CDDP
4-HC

Fig. 3

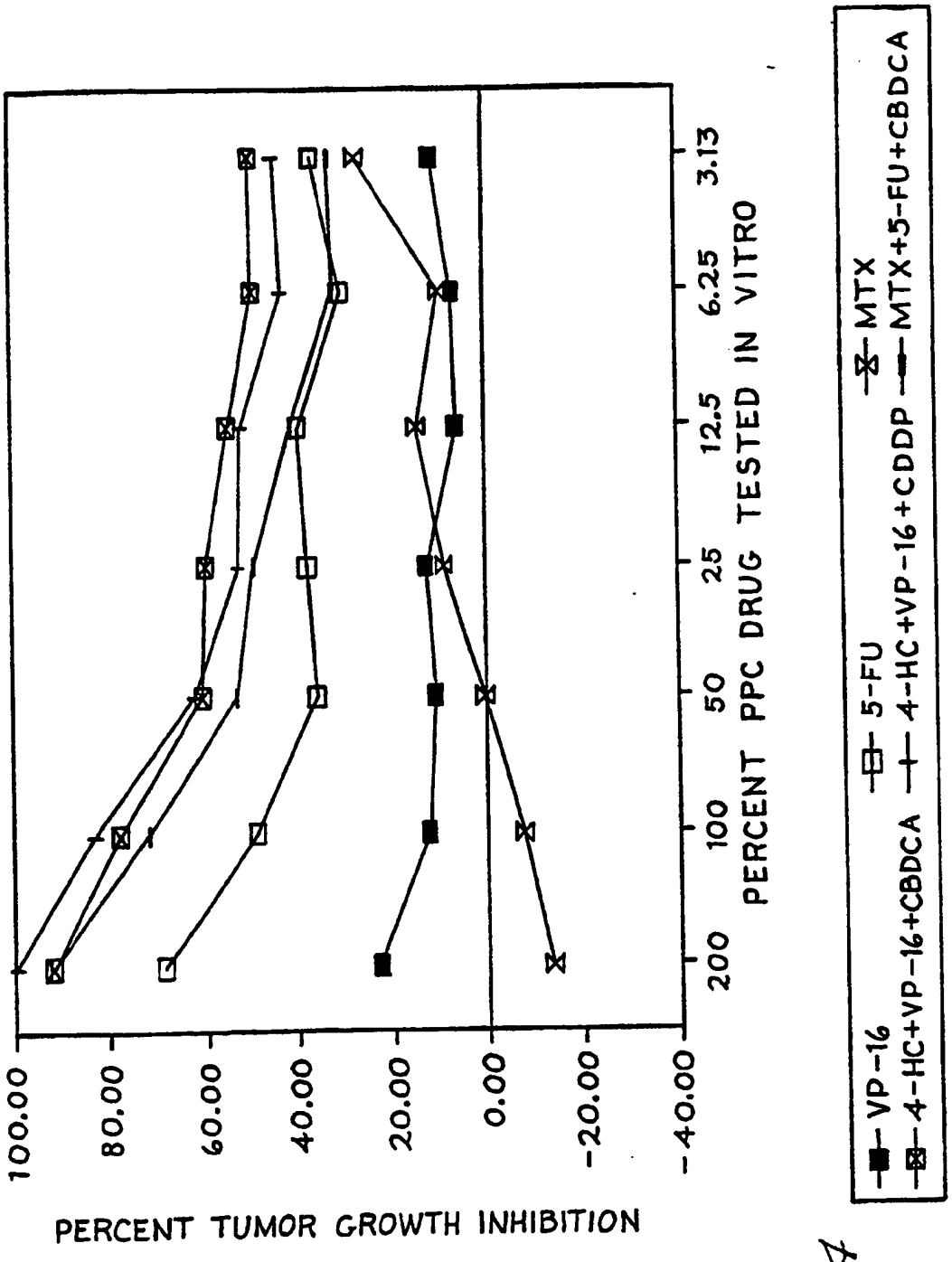

PERCENT TUMOR GROWTH INHIBITION
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
200
100
50
25
12.5
6.25
3.13
PERCENT PPC DRUG TESTED IN VITRO
VP-16
5-FU
MTX
4-HC+VP-16+CBDCA
4-HC+VP-16+CDDP
MTX+5-FU+CBDCA

Fig. 4

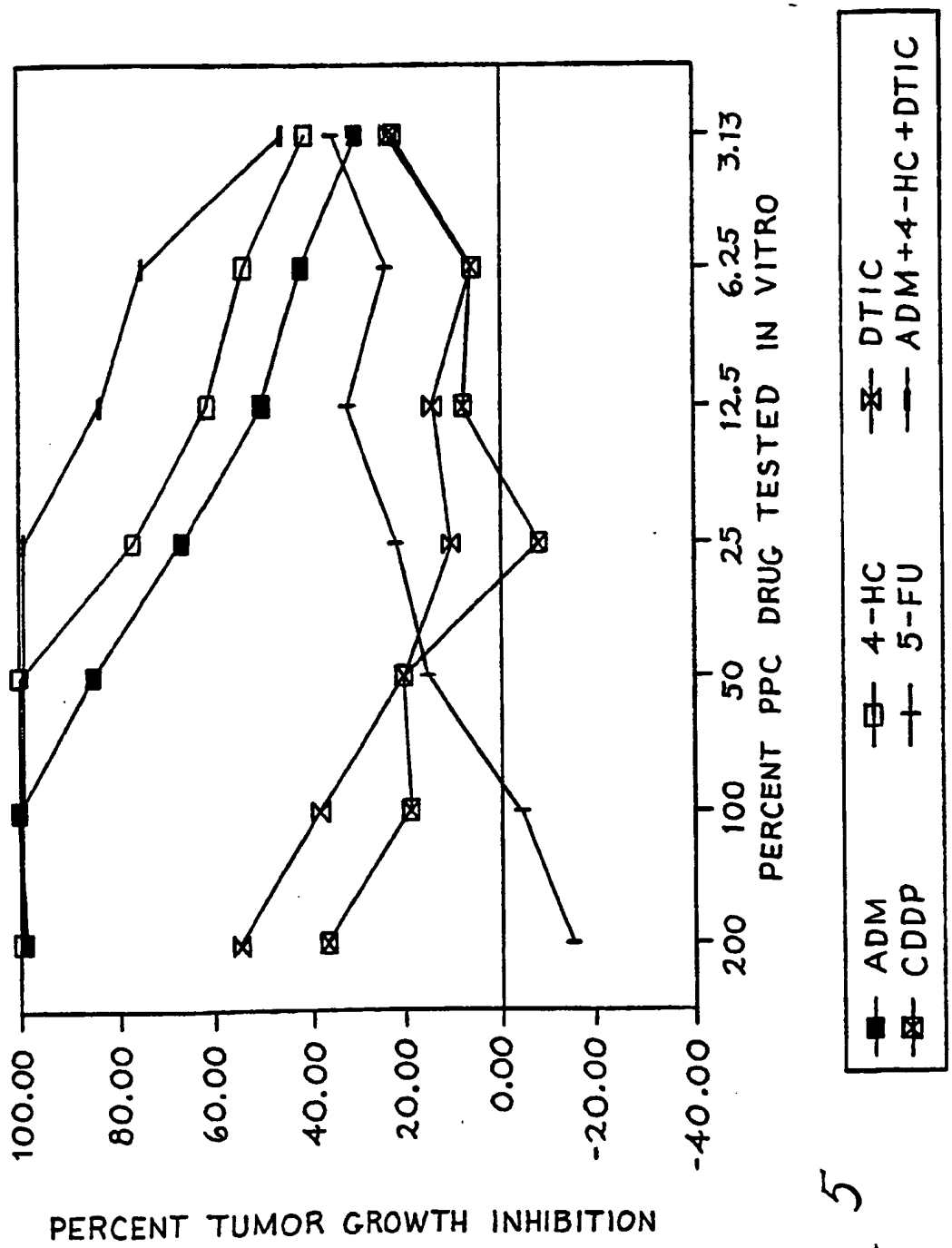

100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
PERCENT TUMOR GROWTH INHIBITION
200
100
50
25
12.5
6.25
3.13
PERCENT PPC DRUG TESTED IN VITRO
ADM
CDDP
4-HC
5-FU
DTIC
ADM+4-HC+DTIC

Fig. 5

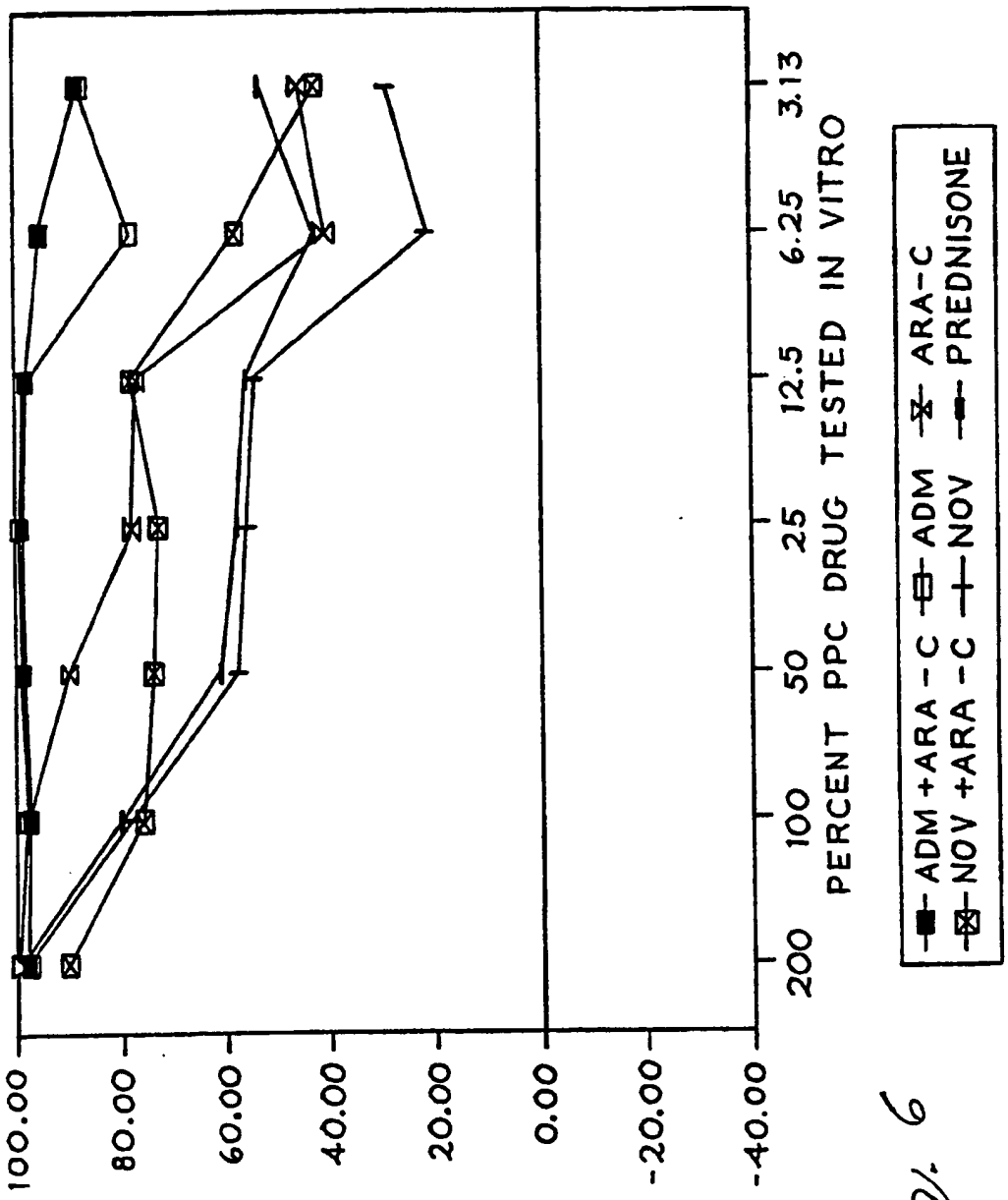

100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
PERCENT TUMOR GROWTH INHIBITION
200
100
50
25
12.5
6.25
3.13
PERCENT PPC DRUG TESTED IN VITRO
ADM +ARA-C
NOV +ARA-C
ADM
NOV
ARA-C
PREDNISONE

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00973

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/00, 5/02; C12Q 1/68, 1/66, 1/02; A61K 31/70

US CL :435/8, 7.9, 6, 29; 436/501, 813; 424/1.1; 128/804, 665

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/8, 7.9, 6, 29; 436/501, 813; 424/1.1; 128/804, 665

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS

Search Terms: cancer agent, ATP, ammonium vanadate, chemotherapy, luminescence, cell culture, serum

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Gynecologic Oncology, Vol. 31, issued 1988, Sevin et al., "Application of an ATP-Bioluminescence Assay in Human Tumor Chemosensitivity Testing", pages 191-204, see entire document.	1-17
Y	US, A, 5,063,157 (Stockinger) 05 November 1991, see entire document.	1-17
Y	The Journal of Biological Chemistry, Vol. 252, No. 21, issued 1977, L.C. Cantley, Jr., et al., "Vanadate is a Potent (Na,K)-ATPase Inhibitor Found in ATP Derived from Muscle", pages 7421-7423, see entire document.	1-17
A	US, A, 4,303,752 (Kolehmainen et al.) 01 Dec. 1981, see entire document.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

28 MARCH 1994

Date of mailing of the international search report

14 APR 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Louise N. Leary Jill Warden for

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	U.S, A, 4,937,182 (Hancock et al.) 26 June 1990, see entire document.	1-17
A	US, A, 4,930,516 (Alfano et al.) 05 June 1990, see entire document.	1-17
A	US, A, 5,049,372 (Rapaport) 17 September 1991, see entire document.	1-17
A,P	US, A, 5,242,806 (Yen-Maguire et al.) 07 September 1993, see entire document.	1-17
A	US, A, 5,005,588 (Rubin) 09 April 1991, see entire document.	1-17